EXHIBIT 3

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Intravesical and Intravenous Therapy of Human Bladder Cancer by the Herpes Vector G207

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ABSTRACT

G207, a conditionally replicating herpes vector, efficiently kills human bladder cancer cells *in vitro*. To evaluate the therapeutic potential of G207, we have established three *in vivo* models similar to the clinical situation. *In vivo*, G207 was intraneoplastically, intravesically, or intravenously inoculated in nude mice. Intraneoplastic inoculation into subcutaneous tumor caused significant tumor growth inhibition. Intravesical inoculation of G207 also caused decreased tumor growth in an orthotopic human bladder cancer model. Furthermore, multiple intravenous inoculation markedly inhibited subcutaneous tumor growth. These results suggest that intravesical therapy with G207 is effective for localized bladder tumor, especially for carcinoma *in situ* (CIS), and intravenous therapy with G207 is promising for invasive or metastasized bladder tumor.

OVERVIEW SUMMARY

G207 is an oncolytic replication-competent herpes simplex virus (HSV) with deletions in the γ34.5 gene and a *lacZ* insertion in the ICP6 gene. G207 shows the potential for antitumor activity against nonneural human tumors as well as neural tumors. In this article we examine the antitumor activity of G207 against human bladder cancer cell lines *in vitro* and *in vivo*. Our report has shed new light on viral therapy using the oncolytic virus G207. This therapeutic strategy appears to be promising as a less invasive treatment for both localized cancer such as CIS and metastasized bladder cancer.

INTRODUCTION

BLADDER CANCER is a commonly occurring cancer. More than 54,000 new cases have been diagnosed in 1999 and approximately 12,000 persons would die of this disease this year (Landis *et al.*, 1999). Transurethral resection of tumor (TUR) is considered to be the most effective therapy for the management of superficial bladder cancer. However, bladder cancer sometimes spreads widely in the mucosa without making an exophytic lesion, called carcinoma *in situ* (CIS). For the treatment of CIS, intravesical instillation with bacillus Calmette-Guérin (BCG) has been widely used, although the side effects, such as urinary frequency, urgency, hemorrhagic cystitis, and fever, often make it difficult to continue this therapy. It is also possible that some part of the CIS becomes invasive bladder carcinoma. Approximately 25% of newly diagnosed bladder cancers have muscle invasions and after radical cystectomy half of them will recur with distant metastases within 2 years (Prout *et al.*, 1979), and long-term survival is rare. We have explored the use of the conditionally replicating herpes vector G207 in bladder cancer models. G207 has several important advantages, as described previously (Yazaki *et al.*, 1995; Mineta *et al.*, 1995), and the cytopathic effect of G207 is limited to neoplastic cells.

In this study we demonstrate that G207 is effective via local or intravenous delivery for the treatment of both localized and metastasized bladder cancer.

MATERIALS AND METHODS

Cells and viruses

Human transitional cell carcinoma cell line KU19-19 and T24 were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (IFBS) at 37°C in humidified 5% CO_2 with penicillin and streptomycin (Sigma, St.

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Louis, MO). African green monkey kidney cells (Vero cells) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% IFBS. Viral stocks of G207 were generated from low multiplicities of infection (MOIs) of Vero cells at 37°C (Mineta *et al.*, 1995). The virus was titered by a plaque assay on Vero cells as described previously (Martuza *et al.*, 1991).

Cell culture cytotoxicity

KU19-19 and T24 cells (1×10^6) were plated in six-well dishes, 24 hr before virus infection. The cells were infected with G207 at an MOI of 0.1 plaque-forming unit (PFU)/cell, while the controls were mock infected with the extract, prepared from mock-infected cells by the same procedure as used for virus inoculum. The viable cell numbers were determined by trypan blue exclusion. All assays were performed in triplicate.

Single-step viral growth

Subconfluent monolayers of KU19-19 and T24 in six-well dishes were infected at an MOI of 0.1 in 0.7 ml of phosphate-buffered saline (PBS) supplemented with 1% IFBS at 37°C in 5% CO_2. Three, 6, 12, 24, and 48 hr after infection, virus was harvested from the wells and titration was performed. Plaques were counted and expressed as plaque-forming units per milliliter. PFU/ml.

Animal studies and X-Gal staining

Six-week-old female athymic BALB/c nude mice (*nu/nu*) purchased from Japan SLC (Shizuoka, Japan) were kept in groups of five or fewer, housed in sterile cages, and had free access to autoclaved food and water. All animal procedures were approved by the Laboratory Animal Center (School of Medicine, Keio University, Tokyo, Japan). Regarding the surgical procedures, each mouse was anesthetized with an intraperitoneal 0.25-ml injection of a solution consisting of 84% bacteriostatic saline, 10% sodium pentobarbital (50 mg/ml; Abbott Laboratories, Chicago, IL), and 6% ethyl alcohol. Mice were visited daily to check their viability status.

Model 1: Subcutaneous tumor model and intraneoplastic inoculation into subcutaneous tumor

The subcutaneous tumors were induced by right flank injection of 1×10^6 KU19-19 and T24 cells in 100 μl. Mice harboring subcutaneous tumors (approximately 5 mm in diameter) were randomly divided ($n = 5$ per group) and treated intraneoplastically with either 1×10^7 PFU of G207 suspended in 40 μl of virus buffer or with 40 μl of mock-infected extract. The tumors were measured by external caliper measurements to within 0.1 mm. Serial tumor volume was obtained by bidimensional diameter measurements and the tumor growth ratio was determined as $0.5(a \times b^2)_{\text{day n}}/0.5(a \times b^2)_{\text{day 0}}$, where a is the longer axis and b is the shorter axis. Animals were killed when the tumor diameter was greater than 18 mm. Statistical differences in growth ratio were assessed by using an unpaired t test.

For pathological studies, the mice bearing tumors (greater than 10 mm in diameter) were treated with an intraneoplastic inoculation of 1×10^7 PFU of G207 and killed on day 5 postinjection. The tumor specimens were fixed in 0.5% paraformaldehyde (Nacalai Tesque, Tokyo, Japan) and 0.5% glutaraldehyde (Nacalai Tesque) in PBS for 24 hr at 4°C. The tumors were then placed in a substrate solution containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 1 mg/ml; Takara,

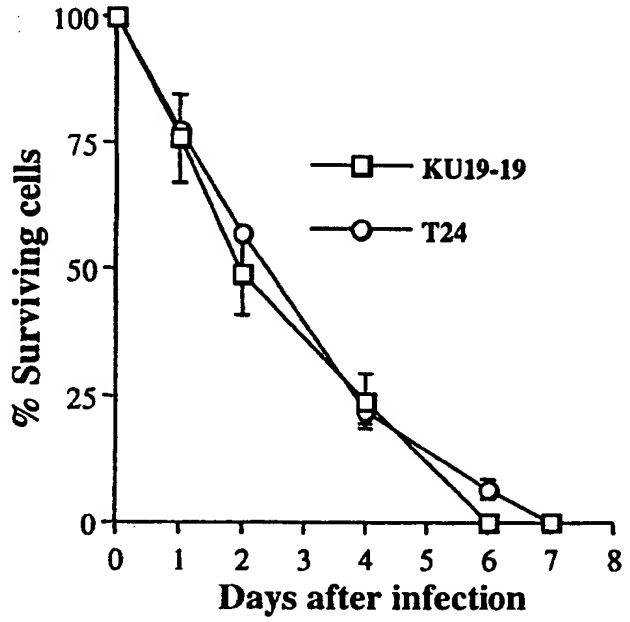

FIG. 1. Susceptibility of human bladder cancer cell lines to G207 *in vitro*. KU19-19 (□) and T24 (○) cells were infected with G207 at an MOI of 0.1. The data plotted are the means of triplicate wells.

Kyoto, Japan), 5 m*M* potassium ferricyanide, 5 m*M* potassium ferrocyanide, and 2 m*M* magnesium chloride in PBS for 6 hr at 37°C, washed with PBS, and incubated overnight in cold PBS containing 30% sucrose. After being frozen in O.C.T. compound (Miles, Elkhart, IN) in liquid nitrogen, the tumors were sectioned (8 mm) on a cryostat. The sections were mounted onto gelatin-coated glass slides. The slides were washed with PBS, stained with X-Gal again overnight, and then were counterstained with hematoxylin and eosin solution.

Model 2: Subcutaneous tumor model and intravenous inoculation of the virus

KU19-19 tumors were removed aseptically from the flanks of the host mice, minced into 1-mm pieces, and transplanted into other mice for study. Mice harboring subcutaneous tumors (approximately 3.5 mm) were randomly divided into two groups ($n = 7$ per group) and inoculated via the tail vein with either G207 (7×10^7 PFU in 100 μl) or mock extract (100 μl) at 0, 2, and 4 days. The tumor growth ratio was assessed as described above. For pathological studies, the mice bearing tumors (greater than 10 mm in diameter) were treated with a single intravenous injection of 7×10^7 PFU of G207 in a volume of 100 μl by the tail vein, and were killed on day 5 postinjection. The tumor specimens were stained with X-Gal as described above.

Model 3: Orthotopic human bladder tumor model and intravesical inoculation into the tumored bladder

An ~5-mm skin incision was made transversely in the abdomen, just above the pubis. The anterior abdominal wall muscles were incised and the bladder was exteriorized. Urine was evacuated by aspiration. Using a 27-gauge needle, a 50-μl suspension of serum-free RPMI 1640 medium containing 10^6 cultured KU19-19 cells was injected into the lumen of the bladder. Mice remained anesthetized for an additional hour to prevent voiding of the tumor cells. Bladder tumors grew and became demonstrable in all mice in 4 days. Four days after tumor implantation, mice were randomly placed into eight groups ($n = 5$ per group), and the bladder of each mouse was exteriorized again. Intravesical infection with G207 (1×10^7 PFU) or mock infection was performed into the empty bladder in a volume of 50 μl. As they awoke, mice were given additional anesthetic to ensure that G207 or mock extract remained in the bladder for at least 60 min. Mice were deeply sedated for more than 1 hr. Tumor incidence and tumor weight, defined as the weight of the tumored bladder, were determined on days 5, 10, 15, and 20 after intravesical therapy, at which time all mice were killed. Statistical differences in the weights of tumored bladders were assessed by using an unpaired *t* test. Whole bladders were removed, stained with X-Gal, and then counterstained with hematoxylin and eosin solution as described above.

RESULTS

In vitro *cytopathic effect*

The susceptibility of human bladder cancer cell lines (KU19-19 and T24) to G207 was evaluated. KU19-19 and T24 cells were efficiently killed by G207 (MOI of 0.1) within 6 and 7 days, respectively (Fig. 1). The cytopathic effect appeared on

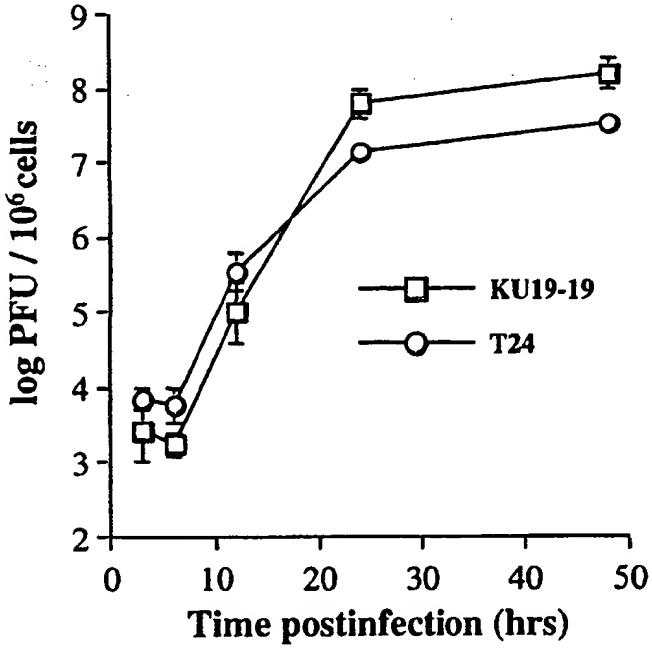

FIG. 2. Viral growth curves for G207 in human bladder cancer cells *in vitro*. KU19-19 (□) and T24 (○) cells were infected with G207 at an MOI of 0.1. Virus was harvested at the time points indicated and titrated on Vero cells. The data plotted are the means of triplicate wells.

day 1 postinfection in both KU19-19 and T24 cells, and effective cytotoxicity with >99% cell destruction was evident on day 6 (KU19-19) and day 7 (T24).

Growth characteristics of G207 in culture

The ability of G207 to replicate in KU19-19 and T24 cells was examined by a viral single-step growth analysis (Fig. 2). The titers were exponentially increased. In KU19-19 cells, the viral yield 24 hr after infection was $10^{7.8}$ PFU/10^6 cells, and the viral yield 48 hr after infection was 10^4- to 10^5-fold greater than those at 3 hr after infection. In T24 cells, the viral yield 24 hr after infection was $10^{7.1}$ PFU/10^6 cells, and the viral yield 48 hr after infection was 10^2- to 10^3-fold greater than those at 3 hr after infection.

Intraneoplastic inoculation in subcutaneous tumors

Xenograft tumors were established in the subcutaneous tissue of the flank of BALB/c (*nu/nu*) mice, using KU19-19 and T24 cell lines. Once the tumors had reached approximately 5 mm in diameter, 1×10^7 PFU of G207 or mock extract was injected intraneoplastically into the flank tumors. From day 2 postinoculation onward the tumor size of both KU19-19 and T24 between G207-treated and control groups diverged, respectively (Fig. 3). When the experiment was terminated on day 14 because of the large tumor burden (>18 mm in diameter) in the control animals, the mean tumor growth ratio was significantly inhibited by G207 (KU19-19: $p < 0.0005$ versus controls, unpaired t test; T24: $p < 0.0005$ versus controls), when compared with control tumor treated with mock extract. The

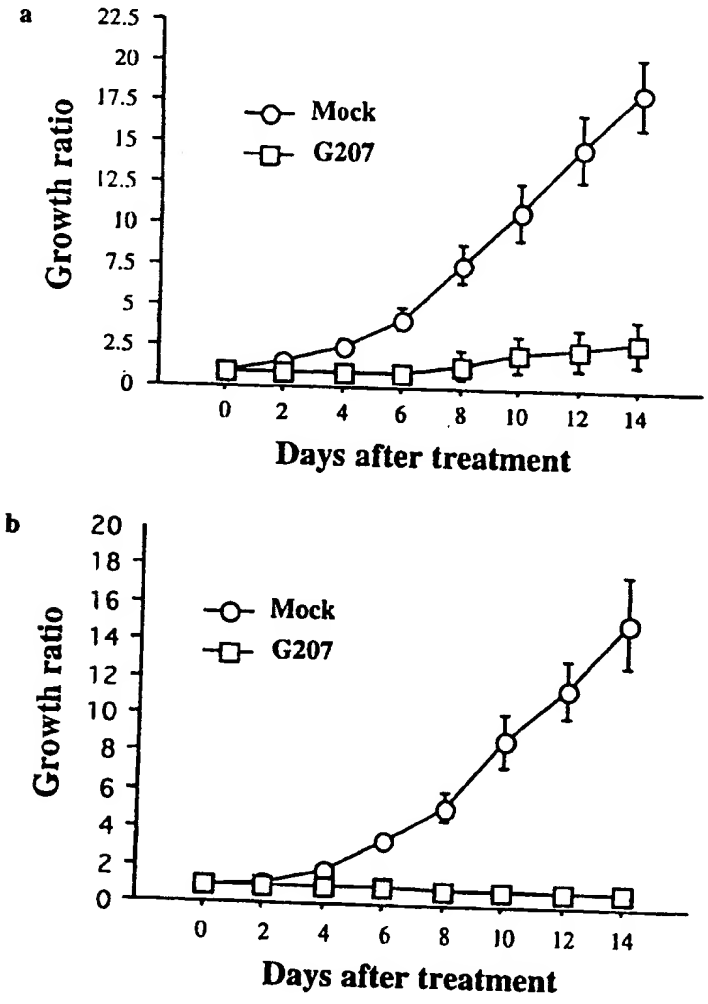

FIG. 3. The intraneoplastic inoculation of G207 in subcutaneous tumor-bearing BALB/c (*nu/nu*) mice. Mice harboring subcutaneous KU19-19 (a) or T24 (b) tumors were treated on day 0 with either 1×10^7 PFU of G207 (□) or mock solution (○). Data represent mean tumor growth ratio ± the standard error of the mean. The subcutaneous KU19-19 (c) and T24 (d) tumors treated with G207 were fixed on day 5, and stained with X-Gal solution. Bar, 1 mm; diffuse β-galactosidase expression is widely seen in both tumors.

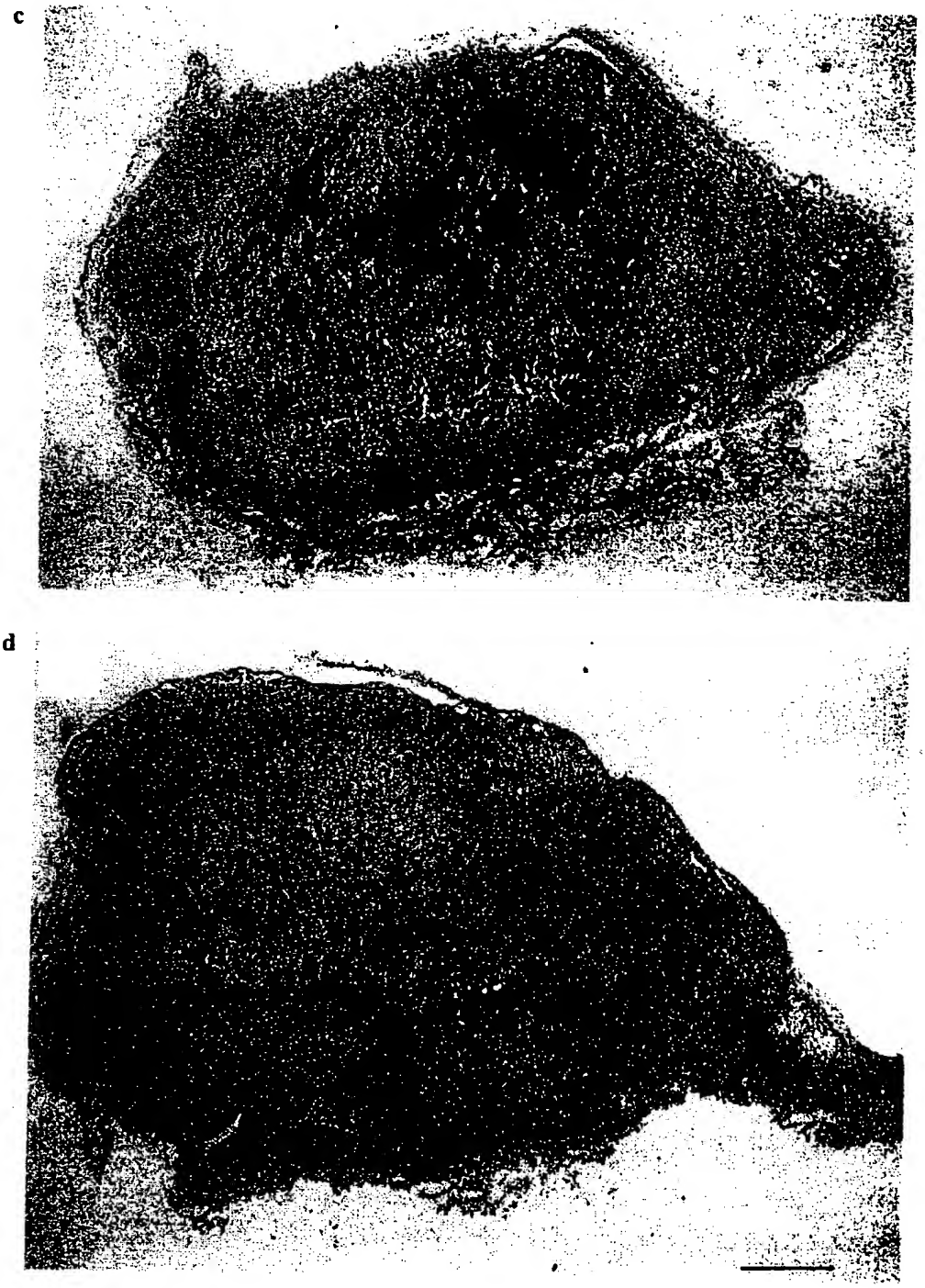

FIG. 3. *Continued.*

growth ratio in G207-treated tumors on day 14 was 2.94 ± 1.37 (SEM) in KU19-19, and 0.781 ± 0.079 in T24, while that in control tumors was 18.281 ± 2.153 and 15.347 ± 2.370, respectively.

To assess the spread of G207 in KU19-19 and T24 tumors *in vivo*, the mice bearing tumors (>10 mm in diameter) were treated with 1×10^7 PFU of G207 and killed on day 5 postinjection. These tumors were stained with X-Gal to examine the extent of β-galactosidase expression. Mock-infected tumors showed no β-galactosidase expression (data not shown). Both

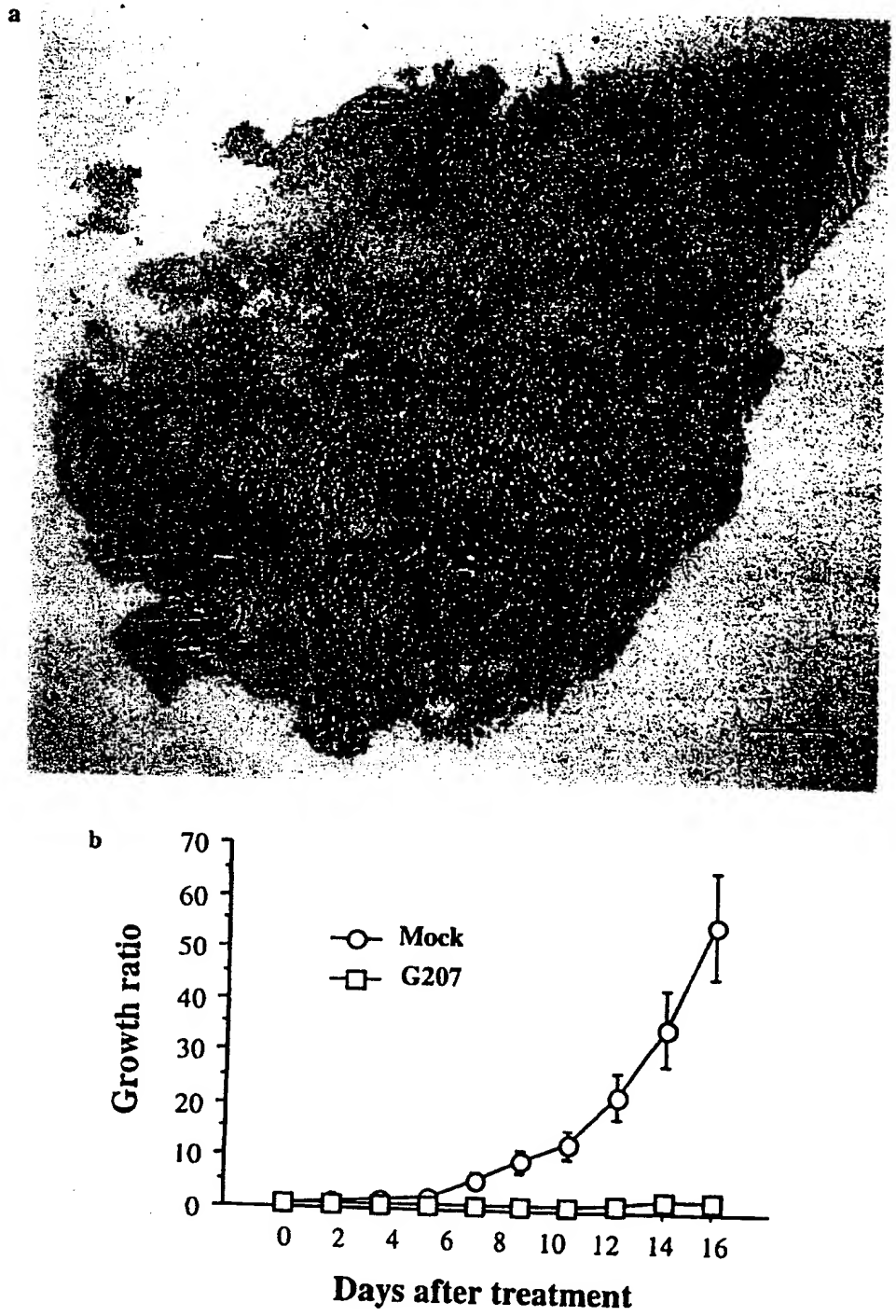

FIG. 4. The effect of intravenous injection of G207 into subcutaneous tumors in BALB/c (*nu/nu*) mice. (a) Pathological examination of G207-treated subcutaneous KU19-19 tumors. Bar, 1 mm; diffuse areas of β-galactosidase expression are sporadically noted. (b) KU19-19 subcutaneous tumors were established in BALB/c (*nu/nu*) mice. Inoculation of 7×10^7 PFU of G207 (□) or mock solution (○) by the tail vein was performed on days 0, 2, and 4. Data represent the mean tumor growth ratio ± the standard error of the mean.

G207-treated KU19-19 (Fig. 3c) and T24 (Fig. 3d) tumors showed the extent of β-galactosidase expression.

Intravenous inoculation in subcutaneous tumors

To examine the possibility of intravenous delivery of G207, a preliminary study was performed. KU19-19 tumors were established in the flank in athymic mice. Once the tumor had reached approximately 3.5 mm in diameter, G207 (7×10^7 PFU/100 μl) or mock extract was administered intravenously through the tail vein. On day 4 after administration of G207 or mock extract, mice were killed and subcutaneous tumors were stained with X-Gal. Diffuse areas of *lacZ* expression were sporadically noted in the G207-treated tumors (Fig. 4a), while none was observed in the tumors treated with mock extract (data not shown). No *lacZ* expression was noted in liver, lung, or kidney on day 4. Therefore, we next examined if the intravenous inoculation of G207 causes the effective tumor growth regression. Either G207 (7×10^7 PFU) or mock extract was administered at 0, 2, and 4 days by tail vein injection. For 3.5-mm tumor fragments, there was no significant difference in tumor volume between G207-treated and control tumors for the first 2 days after intravenous inoculation. From day 4 onward the tumor volume between the two groups diverged (Fig. 4b). On day 18 when the experiment was terminated because of the large tumor burden (>18 mm in diameter) in the control animals, the mean tumor growth ratio was significantly different between G207-treated and control groups ($p < 0.0005$; unpaired t test). The growth ratio in G207-treated tumors on day 18 was 2.590 $\pm$ 1.224 (SEM), while that in control tumor treated with mock extract was 55.387 $\pm$ 10.333.

Intravesical inoculation in orthotopic human bladder tumors

BALB/c (*nu/nu*) female mice ($n = 20$) were given a single intravesical injection with KU19-19 (1×10^6) cells. Aggressive growth of KU19-19 was evident as early as 4 days after injection of tumor cells into the bladder in all tumor-challenged animals. On histological analysis of hematoxylin and eosin (H&E)-stained cells on day 4 after injection, tumor cells showed morphological changes, a disrupted epithelial surface, and an atypical chromatin pattern (Fig. 5a). To evaluate the effect of intravesical treatment, 1×10^7 PFU of G207 was intravesically injected into the lumen of bladders containing tumors on day 4 after implantation of KU19-19 cells. The tumored bladder weight, as a parameter of tumor growth, was determined. The mean tumored bladder weight in mice treated with G207 was significantly reduced when compared with the control on days 5, 10, 15, and 20 after intravesical treatment ($p < 0.005$, unpaired t test; at every time point) (Fig. 5b). On day 20 after intravesical treatment, the mean tumored bladder weight in G207-treated and control mice was 332 $\pm$ 110.861 mg (SEM) and 1386.200 $\pm$ 203.248 mg, respectively. On day 5 posttreatment, positive X-Gal-staining cells were observed at the area consistent with bladder tumors (Fig. 5c). On day 15 posttreatment, tumors were almost gone and a small, sporadic amount of β-galactosidase expression remained (Fig. 5d). No β-galactosidase expression was noted in the control tissues at any time (data not shown).

DISCUSSION

Toxicity and incomplete efficacy of the agents commonly used for the treatment of localized or advanced bladder tumor have prompted a search for alternative treatments (Lee *et al.*, 1994; Ratliff *et al.*, 1998). Bladder tumors are appealing targets for gene therapy protocols because of the ease of access to them via the urethra.

A conditionally replicating herpes vector, G207 (Mineta *et al.*, 1995), has been utilized in treating neural and nonneural malignant tumors (Mineta *et al.*, 1995; Yazaki *et al.*, 1995; Toda *et al.*, 1998, 1999; Carew *et al.*, 1999; Kooby *et al.*, 1999; Walker *et al.*, 1999; Hoshi *et al.*, 2000). We herein demonstrate the efficiency of G207, administered intravesically or intravenously, in treating several stages of bladder tumors. The human-derived bladder cancer cells were susceptible to G207 and G207 progeny widely propagated in those cells *in vitro*. Intraneoplastic inoculation of G207 significantly reduced tumor growth in a subctuaneous model in athymic mice. Furthermore, for the first time, we report a treatment strategy using oncolytic virus in orthotopic human bladder cancer in athymic mice. Intravesical treatment with G207 demonstrated significant tumor growth inhibition from day 5 postinoculation onward. The advantage of this strategy, using conditionally replicating vector, is that all the tumor cells contacting G207 may be killed, and even tumor cells that do not contact G207 stand a good chance of contacting the progeny of G207 released from other G207-infected cells by cytolysis. In this model, G207 therapy has been shown to be promising for localized bladder tumors, especially for CIS. Most CIS are aneuploid grade 3 and the occurrence of CIS could be considered a sign of high aggressiveness. Therefore, bladder CIS is considered one of the most adequate candidates for G207 treatment (Gustafson and Tribukait, 1985; Norming *et al.*, 1992).

For treatment of advanced bladder tumors with/without metastatic lesions, we attempted the systemic approach, inoculating G207 intravenously. Previous studies showed that localized vascular delivery of G207 is effective in experimental hepatic tumors (Kooby *et al.*, 1999) and gliomas (Ikeda *et al.*, 1999), and intravenous administration of G207 induced tumor growth inhibition in an experimental subcutaneous model with prostate cancer cells (Walker *et al.*, 1999). We used subcutaneous flank tumors as a distant metastasis model of bladder cancer, and herein present data indicating the wide distribution of G207 infection by intravenous delivery. Intravenous inoculation is dramatically effective in inhibiting the growth of rapidly growing bladder tumors such as KU19-19. These results indicate that the intravenous delivery of G207 is useful, even for the treatment of a distant metastatic site. However, several problems remain to be considered for systemic delivery of G207. Clinical recipients are immunocompetent, and approximately 90% of the adult population greater than 40 years old is seropositive for herpes simplex virus (HSV) (Corey and Spear, 1986). However, one study showed that there is no difference in the efficacy of G207 tumor therapy administered as multiple intratumoral injections to HSV-seropositive and seronegative tumor-bearing mice (Chahlavi *et al.*, 1999). They suggest that multiple injections of G207 might contribute to any inhibitory effect of induced humoral and cell-mediated immunity to HSV. Therefore, multiple intravenous injections of G207 over a short pe-

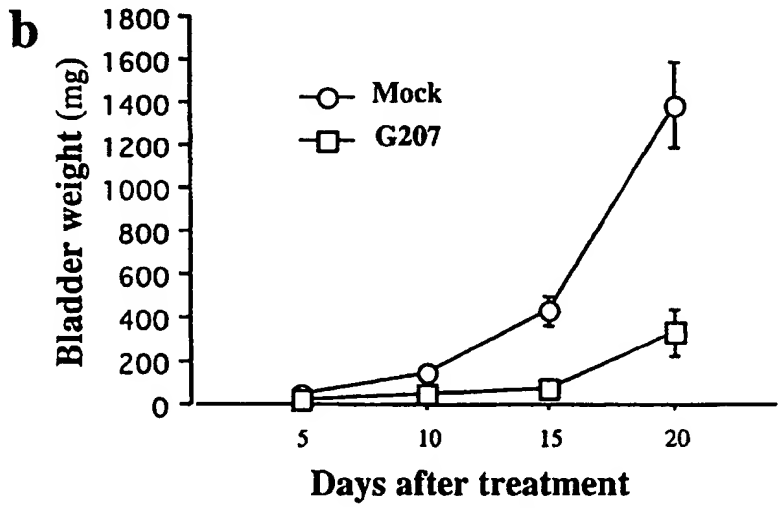

FIG. 5. The effect of intravesical injection of G207 on orthotopic human bladder tumors (KU19-19) in BALB/c (*nu/nu*) mice. (a) Pathological examination of tumored bladder on day 4 after injection of KU19-19 cells. Tumored bladder was fixed and stained with H&E. Bladder tumor was arising, and presented the characteristic change in morphology. (b) Bladder tumors were treated with either 1×10^7 PFU of G207 (□) or mock solution (○). Data represent mean tumor volume ± the standard error of the mean. Mice were killed on days 5, 10, 15, and 20, and tumored bladder weight, as a parameter of tumor growth, was determined. (c and d) Pathological examination of tumored bladder on day 5 (c) and day 15 (d) after treatment with 1×10^7 PFU of G207. Tumored bladders were fixed and stained with X-Gal solution. On day 5 (c) positive X-Gal-staining cells were observed in the areas consistent with bladder tumors. On day 15 (d), tumors were almost alleviated, and a small number of positive X-Gal-staining cells were observed. (a, c, and d) Magnification bar: 1 mm.

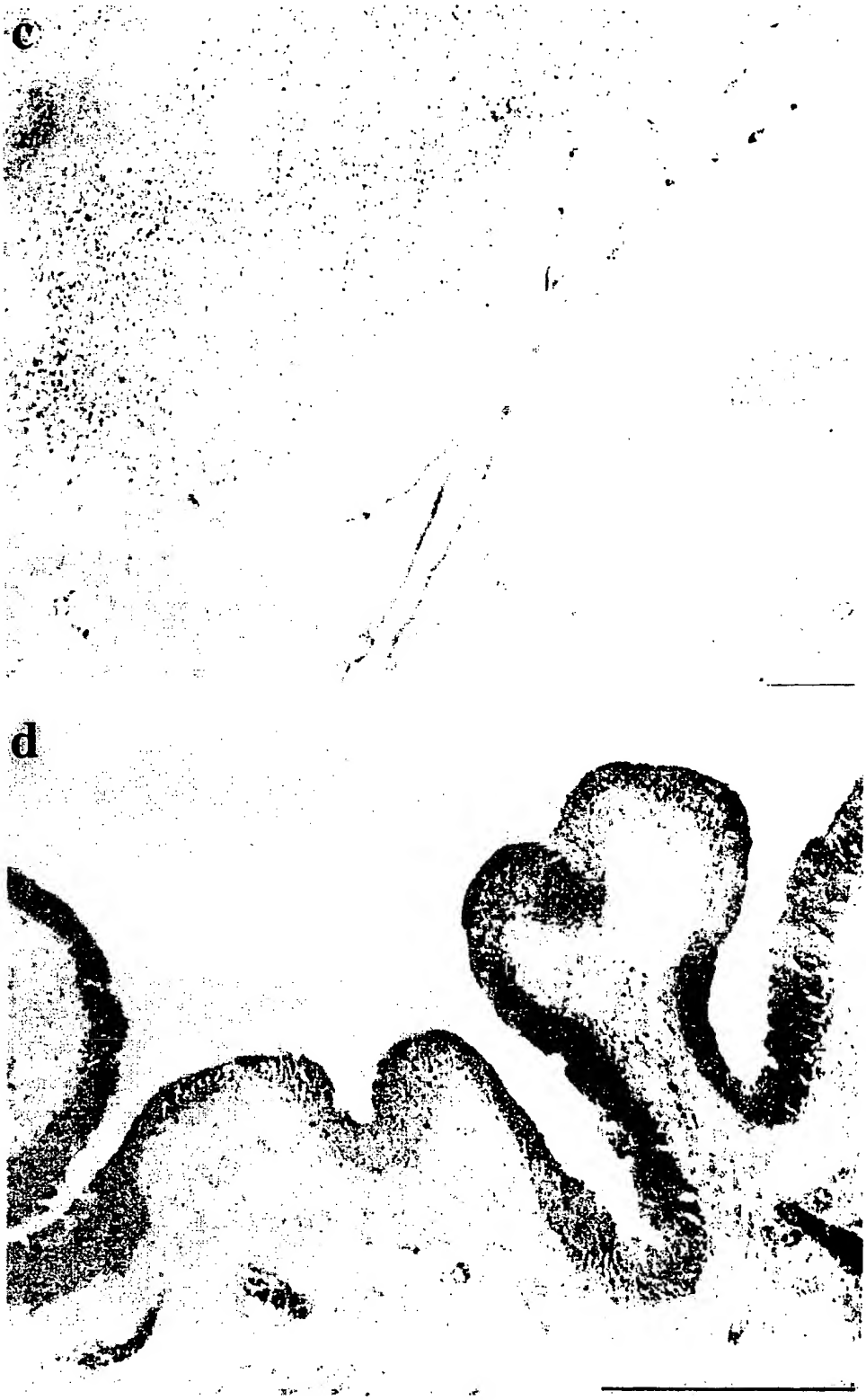

FIG. 5. *Continued.*

riod may be an appropriate strategy for invasive bladder tumors with or without distant metastasis. Concerning whether intravenous delivery is safe for normal organs, a previous study showed that intracerebral injection of G207 in nonhuman primates was nontoxic, and even animals with humoral immunity due to prior exposure to G207 experienced no adverse consequences (Hunter *et al.*, 1999). Further, the intravenous inoculation of 1×10^7 PFU of G207 was not toxic in BALB/c mice (Sundaresan *et al.*, 2000). We also confirmed that the liver, kidney, and lung of athymic mice were not damaged when 7×10^7 PFU of G207 was delivered intravenously (data not shown). Therefore, these results suggest that local or systemic inoculation of G207 is safe and would be worth considering for human clinical trials.

In addition, one point in our study concerns the expression of the coxsackie and adenovirus receptor (CAR), thought to increase infection by cytolytic adenoviruses, by bladder cancer cells. One report demonstrated that several commonly used bladder cancer cell lines, including T24, displayed reduced levels of the CAR and low sensitivity to infection by adenovirus. Our results showing that T24 cells were readily infected by G207, therefore, would seem to be an advantage for herpesvirus vectors over adenovirus vectors for bladder cancer gene therapy (Li *et al.*, 1999).

In conclusion, the present study has focused on the therapeutic potential of intravesical or intravenous administration of G207 as a novel oncolytic strategy against bladder tumor. Intravesical therapy with G207 seems to be effective for localized bladder tumor, especially for CIS. Intravenous therapy with G207 would also be promising for invasive or metastasized bladder tumor. Therefore, we encourage human trials of these therapies in the near future although further safety evaluation, especially on systemic administration of G207, will be needed. We believe oncolytic viruses will in the future reliably alleviate several types of malignant tumor.

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